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Developmental Expression of Kv Potassium Channels Segment of Cultured Hippocampal Neurons

Diana Sánchez-Ponce, Javier DeFelipe, Juan José Garrido , Alberto Muñoz

Abstract

Axonal outgrowth and the formation of the axon initial segment (AIS) are early events in the acquisition of neuronal concentration of voltage-dependent sodium and potassium channels. However, the specific ion channel subunits pr subdomain vary both during development and among the types of neurons, probably determining their firing charac characterize the developmental expression of different subfamilies of voltage-gated potassium channels in the AIS including subunits Kv1.2, Kv2.2 and Kv7.2. In contrast to the early appearance of voltage-gated sodium channels a subunits were tethered at the AIS only after 10 days *in vitro*. Interestingly, we observed different patterns of Kv1.2 to distinct neuronal populations. The accumulation of Kv1.2 and Kv2.2 subunits at the AIS was dependent on anky the actin cytoskeleton and it was resistant to detergent extraction, as described previously for other AIS proteins. further emphasizes the heterogeneity of this structure in different neuronal populations, as proposed previously, an potential regulation.

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Introduction

The non-uniform distribution of specific of voltage-gated K^+ (Kv) channels and their restriction to discrete neuronal neuronal excitability. Indeed, these channels are believed to influence different properties of neurons, including rest potential (AP) firing pattern, transmitter release and synaptic strength. The importance of Kv channels in neuronal induced by mutations or diseases that disrupt K^+ channel expression, including episodic ataxia and epilepsies [1], [channels are actually complexes made up of four voltage-sensing and pore-forming subunits, each generated from subfamilies (Kv1–12) [7], [8]. These complexes may assemble with auxiliary β subunits what may influence the exp Kv channels [9], [10], [11]. The axon initial segment (AIS) is a neuronal domain that is densely populated by voltag

critical for input integration and action potential generation [12], [13], [14], [15]. In addition to voltage-gated Na^+ (V channels [12], [21], [22], the AIS of neocortical and hippocampal principal cells is characterized by the expression [23], [24], [25], [26]. The distinct subunit composition of these channels confers distinct biophysical properties to tl Together with heterogeneous expression and localization of such channels in the AIS, they are likely to contribute t neuronal populations, and the corresponding differences in AP initiation and/or propagation [17], [18], [19], [20], [2

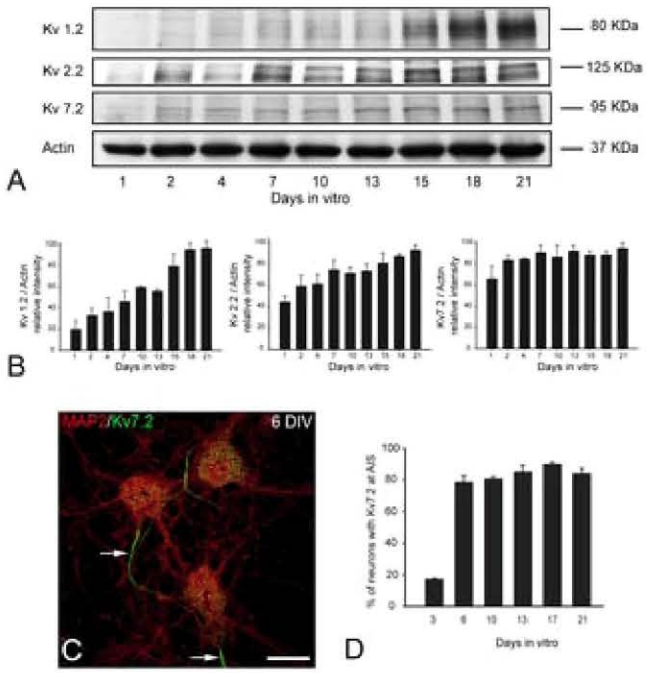

Figure 1. Developmental expression of potassium channels in cultured hippocampal neurons.
(A) Western blot of Kv1.2, Kv2.2 and Kv7.2 in hippocampal neurons cultured at high density (50,000/cm^2) for d conditions. (B) Histograms show Kv1.2, Kv2.2 and Kv7.2 expression normalized to actin when quantified densit the mean ± SE of three independent experiments. Note the delayed onset of Kv1.2 expression as compared w Photomicrograph of hippocampal neurons cultured for 6 DIV and double immunostained for Kv7.2 (green) and in a single process emerging from the cell body (arrows). (D) Histogram shows the percentage (mean ± SE) o different developmental stages *in vitro*. Scale bar = 16 µm.
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Kv1 channels are characterized by low thresholds, as well as rapid activation and slow inactivation kinetics, and th shape and propagation rate of APs, as well as neurotransmitter release and synaptic efficacy [25], [32], [33], [34] regulate somatodendritic excitability and Ca^{2+} influx in hippocampal and cortical neurons during periods of repetitiv [42], [43]. These channels are also localized at the AISs of different neuronal populations, where they contribute tc the inter-spike potential during high frequency firing [44], [45]. Kv7 channels are localized at the AIS [23], [46], [47 M-current. These channels regulate resting potential and AP firing and they are characterized by low-threshold, slc sustained activity and non-inactivation near the AP threshold [47], [52], [53], [54].

Despite the functional importance of the AIS, the timing and the intracellular mechanisms involved in the compartm poorly understood. A key aim of the present study was to describe the distribution, developmental expression and AIS of cultured hippocampal neurons, a model commonly used to study the development of neuronal polarity and a neurons, ankyrin G is one of the earliest markers to be detected at the AIS and it is essential for the tethering of c development, such as VGSC [56], [57], [58]. Like VGSCs, Kv7 channels (Kv7.2 and Kv7.3 subunits) contain a con their targeting to the AIS. Together with the adhesion molecules NrCAM and Neurofascin 186, accumulation of Kv7 interaction of ankyrin G with the actin cytoskeleton via βIV spectrin [16], [46], [48], [49], [56], [57], [59], [60], [61], depends on the structural integrity of actin and on the microtubule cytoskeleton in the AIS [64], [65], [66], [67]. Fur neurons involves the expression of $GABA_A$ receptor subunits and gephyrin clusters [68], as well as the acquisition involved in Ca^{2+} regulation and reaches neurochemical maturation during the second week *in vitro* [69], [70]. To da Kv2 channels in the AIS, along with the associated trafficking and clustering mechanisms, has not been fully elucid the temporal and spatial distribution of Kv1.2 and Kv2.2 subunits during AIS development, and the role of the subn

results show that Kv1.2 and Kv2.2 expression are mutually exclusive in the AIS of cultured hippocampal neurons, a on ankyrin G, yet independent of actin cytoskeleton integrity.

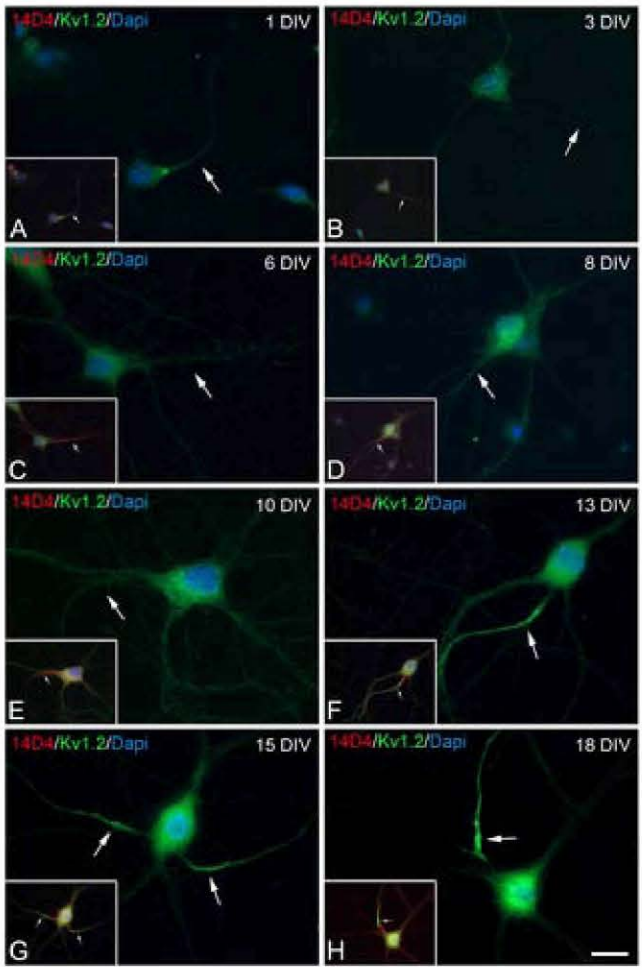

Figure 2. Kv1.2 is concentrated at the AIS during axonal maturation *in vitro*.
Hippocampal neurons were grown for 1, 3, 6, 8, 10, 13, 15 and 18 days at low density (5,000/cm^2), fixed in 4% against Kv1.2 (green) and 14D4 antibodies (red) to identify the AIS. Note that 14D4 staining is detected at the (A, B) and it is restricted to the AIS as the axon elongates (arrows). Confocal microscopy photomicrographs s neurons cultured for up to 10 DIV (A–E). Staining is light and localized to the soma and neurites. After 10 DIV observed in the distal AIS. See Figure 3 for quantification. Scale bar = 18 µm.
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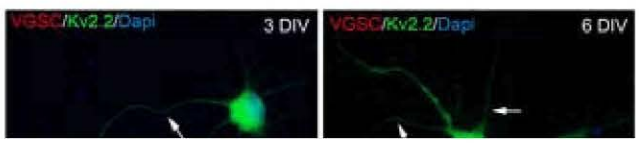
Materials and Methods

Neuronal Cultures

Hippocampal neurons were obtained from E17 mouse embryos and prepared as described previously [55]. Mice v facility. Pregnant female mice and embryos were sacrificed by cervical dislocation and decapitation respectively fo Convention ETS123, recently revised as indicated in the Directive 86/609/EEC. In addition all protocols were appr committee (Subcomité de Bioética, CSIC; Institutional review board; IRB 0007851).



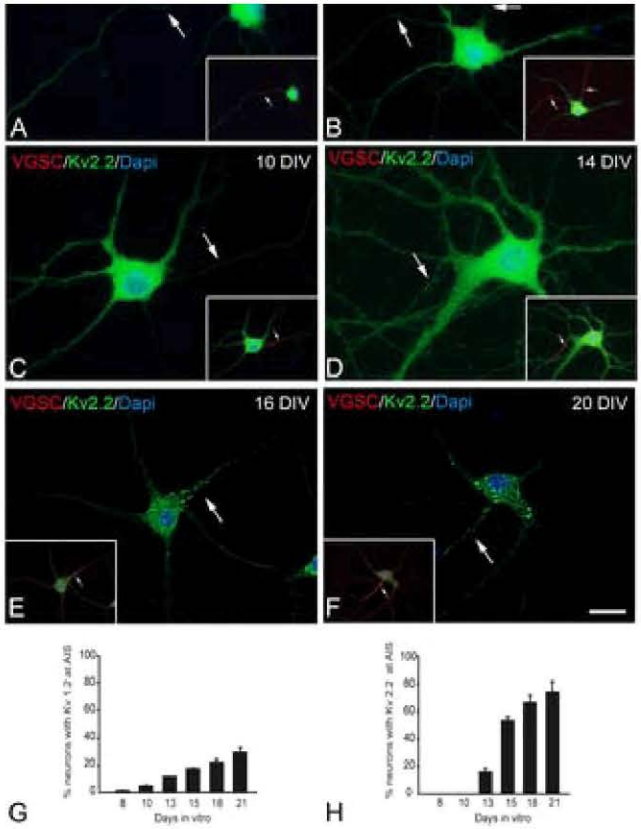


Figure 3. Kv2.2 concentration at the AIS increases during axonal maturation *in vitro*.
Confocal microscopy photomicrographs showing representative hippocampal neurons cultured for 1, 3, 6, 8, 1((5,000/cm^2), fixed in 4% PFA, and stained with antibodies against Kv2.2 (green) and VGSC (red). According to 2008), VGSCs concentrate at the AIS (arrows) after 3 DIV. Note that moderate Kv2.2 immunostaining is locali processes at all developmental stages in culture (A–C). After 14 DIV (D–F), Kv2.2 expression is evident in the proximal region of the AIS. Histogram shows the percentage of neurons expressing Kv1.2 (G) and Kv2.2 (H) at *vitro* (the data represent the mean ± SE from three independent experiments). Scale bar = 18 μm (A–D) and 1
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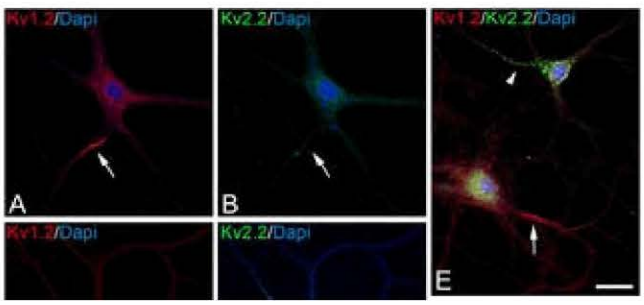
Briefly, after dissection of the hippocampus, tissue pieces were washed three times in Ca^{2+}/Mg^{2+}-free HSBB and 0.2% trypsin. The tissue was washed three times in Ca^{2+}/Mg^{2+}-free HBSS and dissociated with a fire-polished Pa resuspended in plating medium (MEM with 10% Horse Serum and 0.6% glucose) and plated on polylysine coated cm^2 (low density) for immunostaining, or 50,000 cells per cm^2 (high density) for Western blots. After 2 hours, the r medium (Neurobasal medium supplemented with B-27 and glutamax-I). To maintain the neurons for 21 days *in vitr* plates containing astrocyte monolayers that had been cultured in neuronal culture medium for 24 h previously. 1-β- added to the culture after 3 days to prevent astroglial cell growth, and in some cases neurons were treated betwe (Sigma) to impede actin polymerization. For detergent extraction, neurons were maintained in culture for 21 DIV, w minutes at 37°C with 1% Triton X-100 in cytoskeletal buffer (2 mM $MgCl_2$, 10 mM EGTA, 60 mM Pipes [pH 7.0]), experiments, the plasmids were introduced into hippocampal neurons by nucleofection prior to plating (Amaxa Bios manufacturer's instructions. Nucleofection was performed using 3 μg of total DNA and the plasmids used for trans shRNA in a pGFP-V-RS plasmid and a shRNA-AnkG (sequence: TCGGATAGGTCCTACACCTTGAACAGAAG) in USA). The effects of nucleofection were analyzed at 18 DIV.



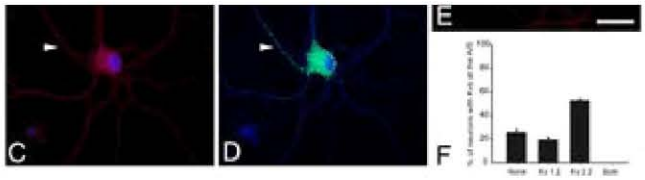


Figure 4. Lack of Kv1.2 and Kv2.2 colocalization at the AIS of cultured hippocampal neurons.
A–B and C–D: Pairs of representative confocal microscopy photomicrographs of hippocampal neurons culturec (red) and Kv 2.2 (green), and counterstained with DAPI (A–D). Note that Kv1.2-expressing AISs (arrows) lack (E). Histogram shows the proportion of neurons expressing Kv1.2, Kv2.2, neither or both at the same AIS at 1 three independent experiments). Scale bar = 25 µm.
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Western Blotting

Protein samples were prepared from hippocampal neurons cultured at high density (50,000/cm^2) in control conditic 21 DIV), the plates were washed twice with cold PBS, the neurons were lysed and then homogenized in a buffer c 100 mM NaF; 1% Triton X-100; 1 mM sodium orthovanadate; 10 mM EDTA; and a complete protease inhibitor coc The lysates were boiled for 10 minutes, separated by SDS-PAGE on 8% acrylamide gels and transferred to nitroc incubated overnight at 4°C with primary antibodies in blocking solution (PBS, 0.2% Tween-20 and 5% BSA): mous rabbit anti-Kv2.2 (1:500; Alomone, Jerusalem, Israel); mouse anti-Kv7.2 (KCNQ2, 1:500; Neuromab) and mouse a After washing, the membranes were incubated with the corresponding peroxidase conjugated secondary antibody was visualized by ECL (Amersham). Densitometry was performed using an imaging densitometer (GS-800, BioRa whole-image background subtraction tool (Quantity One software, BioRad).

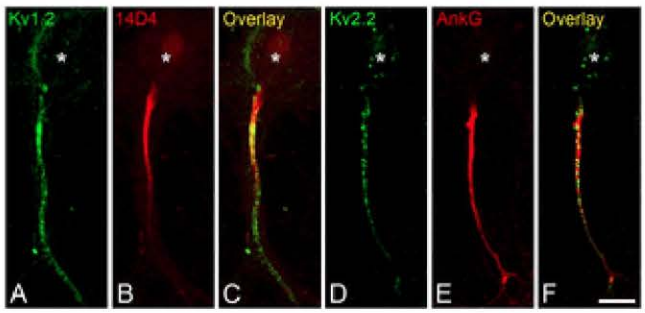

Figure 5. AIS resistance to detergent extraction.
Hippocampal neurons cultured for 15 min in a buffer containing 0.5% Triton X-100 b analyzed by confocal microscopy. After detergent extraction, Kv1.2 (A) and Kv2.2 (D) were still present in the A resistant AIS markers 14D4 (B) and ankyrin G (E), respectively. Asterisks indicate the location of the neuronal
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Immunocytochemistry

After different times in culture, neurons were fixed in 4% paraformaldehyde for 20 minutes and then washed in PB with 50 mM NH_4Cl and incubated in blocking buffer for 45 minutes (PBS, 0.22% gelatin and 0.1% Triton X-100). A were incubated for 1 h at room temperature with the primary antibodies to Kv channel subunits diluted in blocking l anti-Kv1.2 (1:250; Neuromab); mouse anti-Kv 7.2 (KCNQ2, 1:200; Neuromab). To identify the AIS we used mouse mouse anti-ankyrin G (1:200; Neuromab) antibodies and rabbit antibodies (14D4) raised against phospho (p-32)-I recognize an uncharacterized phosphorilated protein present at the AIS [71]. Mouse anti-Tau-1 (1:1,000; Sigma) a Cambridge, UK) antibodies were used to reveal axonal and neuronal morphology respectively. In some neurons, a phalloidin (1:100; Invitrogen, A-12379). The secondary antibodies used were donkey anti-mouse, anti-rabbit or ant 647. After staining coverslips were counterstained with DAPI (1:1000, Calbiochem, San Diego, CA, USA) and mou

Birmingham, AL, USA). Images were obtained using a DP70 camera attached to an Olympus BX51 fluorescence r microscopy (Zeiss 710). Z sections were recorded at 0.2–1-µm intervals through separate channels and ZEN 200 composite images from each optical series by combining the images recorded through the different channels. In al used to generate the figures (Adobe Systems Inc., San Jose, CA, USA). The cell counts in the different experimer t-test using Sigma Plot 11.0 software.

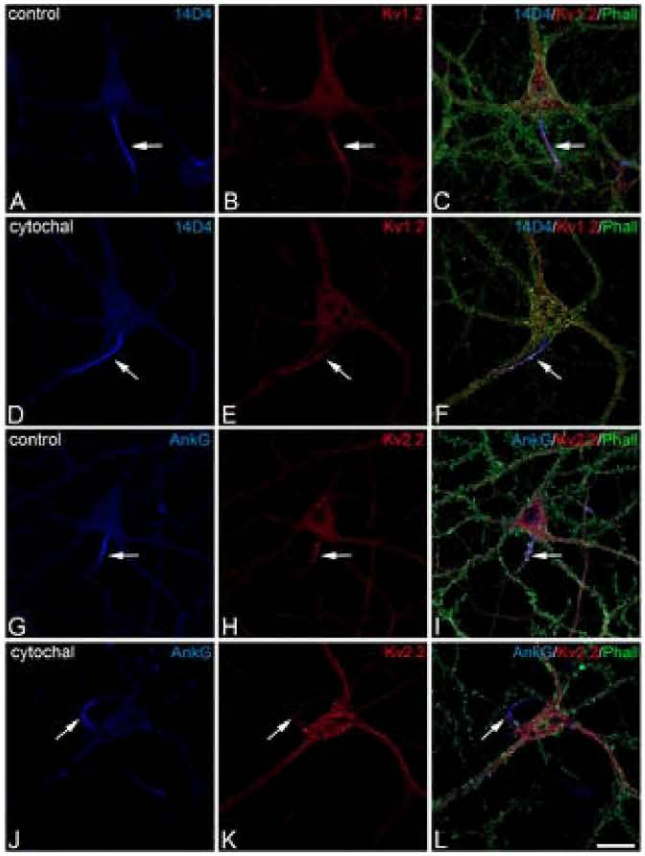

Figure 6. AIS Kv channel expression is not dependent on the actin cytoskeleton.
Confocal microscopy photomicrographs show that Kv1.2 (A–F) and Kv2.2 (G–L) accumulation in the AIS is not neurons were exposed to DMSO (control, A–C, G–I) or cytochalsin D (5 µM; D–F, J–L) from 15 to 17 DIV, dou Kv1.2 (red, A–F) or Kv2.2 (red, G–L), and stained with Alexa 488 phalloidin to reveal F-actin. Note the presenc and cytochalasin D-treated neurons. Scale bar = 25 µm (A–F) and 30 µm (G–L).
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Results

Voltage-gated Potassium Channel Expression in the Developing AIS of Cultured Hi

We first analyzed the expression of voltage-gated potassium channels in hippocampal neurons cultured at high den total expression of the different Kv subunits was analyzed in Western blot and the results were normalized to the e expression was only weakly detected during the first days *in vitro*, yet it increased progressively from about 13 DI expression was clearly evident from the first day in culture and it increased progressively thereafter. The pattern o observed for Kv2.2, consistent with previous studies describing the early onset of Kv7.2 expression at the AIS. Th ankyrin G [48], which is expressed in the developing AIS from 3 DIV [58].

We next used immunocytochemical analysis to study the localization of these voltage-gated potassium channels in neurons. When we studied the localization of Kv7.2 subunit in our cultures (Fig. 1C–D), it was already present in th and from 6 DIV onwards, it was detected in ~80% of neurons: 78.64 (±4.38%) at 6 DIV; 80.88 (±1.39%) at 10 DI 17 DIV; and 84.44 (±2.96%) at 21 DIV. Kv7.2 was distributed homogeneously in the AIS and it was detected alon

development (Fig. 1C). As the expression patterns of Kv7.2 and Kv7.3 at the AIS along with the mechanisms that characterized [48], [50] we subsequently focused on Kv1 and Kv2 channel expression. Accordingly, the results des Kv1 and Kv2 channels towards the axon lags behind that of sodium channels and Kv7.2 channels.

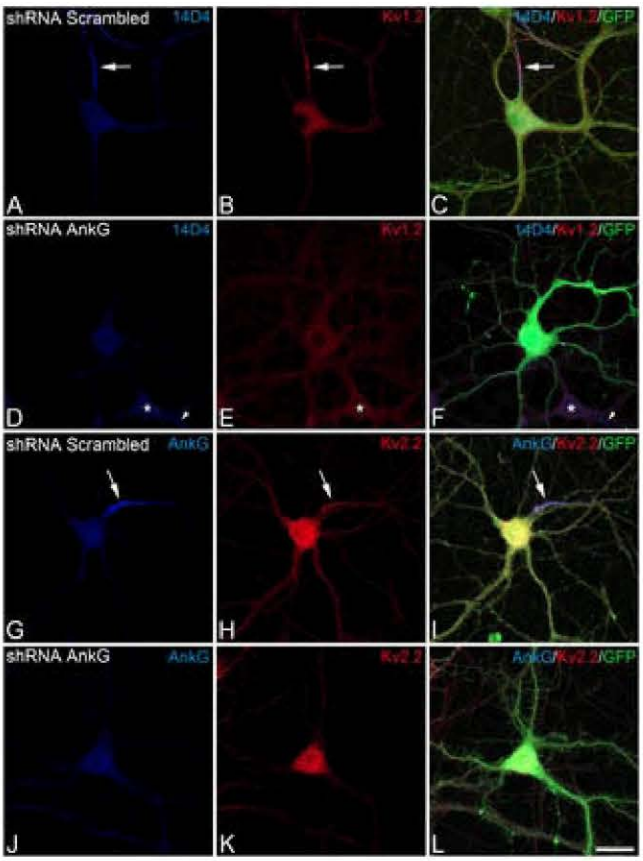

Figure 7. AIS Kv channel concentration is dependent on ankyrin G.
Confocal microscopy photomicrographs show that interference RNAs against ankyrin G impair the concentratic plating, hippocampal neurons were nucleofected with plasmids expressing scrambled shRNA (A–C, G–I) or an cultured until 18 DIV. The neurons were then double stained with antibodies against ankyrin G or pIκBα (14D4 neurons were identified by GFP fluorescence. Ankyrin G, the protein recognized by 14D4 antibodies, Kv1.2 an expressing scrambled shRNA plasmids (A and G) and in non-nucleofected neurons (arrowhead in D–F). Howe nucleofected with ankyrin G shRNA (D–F, J–L), no Kv1.2 or Kv2.2 immunostaining was observed at the AIS. N cell somata were not affected by ankyrin G interference (K). Scale bar = 25 µm. Arrows indicate AISs and ast neurons.
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Kv1.2 Expression in the AIS of Cultured Hippocampal Neurons

To study the expression of low threshold voltage-gated potassium (Kv1) channels during the development of neuro were cultured for different intervals and stained with antibodies raised against the Kv1.2 subunit (Fig. 1). Kv1.2 wa culture, as identified by 14D4 staining, which co-localizes with the axonal marker SMI-31 [58]. In parallel with axon of 14D4 immunostaining in the AIS, whereas diffuse Kv1.2 staining was observed in neurons, predominantly in the AIS until around 8 DIV (Fig. 2 A–E). Consistent with the findings in Western blots, Kv1.2 immunostaining was more of some neurons after 10 DIV (Fig. 2F–H). Indeed, the percentage of neurons exhibiting Kv1.2 staining restricted t of cultured neurons by 21 DIV (n = 3): 1.46 (±0.25%) at 8 DIV; 5.15 (±0.39%) at 10 DIV; 12.1 (±0.07%) at 13 DI DIV; and 29.75 (±1.41%) at 21 DIV. Kv1.2 immunostaining was homogeneous, unclustered and mainly concentrate region in which 14D4 staining was detected, exhibited no Kv1.2 expression at any developmental stage (Fig. 2F–H

Kv2.2 Expression at the AIS of Cultured Hippocampal Neurons

To study the expression of delayed rectifier voltage-gated potassium channel (Kv2) during the development of the intervals were stained with antibodies directed against the Kv2.2 subunit (Fig. 3), and for sodium channels to ident immunostaining was evident from the initial stages of neuronal differentiation. Indeed, immunocytochemistry reveal processes at all times in culture. In addition, after 14 DIV (Fig. 3D–F) there were intense patches of Kv2.2 immun in contrast to the pattern of Kv1.2 expression observed. The proportion of neurons exhibiting clustered and polariz progressively after 14 DIV, representing over 70% of the cultured neurons at 21 DIV (n = 3): 16.32 (±2.19%) at 1(at 17 DIV and 74.57 (±7.36%) at 21 DIV.

Mutually Exclusive Kv1.2 and Kv2.2 Expression in the AIS

The differential localization of low-threshold Kv1.2 (distal) and delayed rectifier Kv2.2 potassium channels (proxima is expressed in a specific region of this structure. Indeed, when we double-stained 21 DIV neurons using antibodie of these subunits at the AIS was mutually exclusive and they were localized in distinct neuronal populations (Fig. 4) 19.47 (±1.85%) of neurons, which in turn exhibited no Kv2.2 immunostaining in the AIS (arrows in Fig. 4). By contr the soma and AIS (52.67±1.34%; arrowheads in Fig. 4) no Kv1.2 immunostaining was evident in the AIS. In our e> neurons exhibiting Kv1.2/Kv2.2 double immunostaining in the AIS. Moreover, a significant percentage of neurons e> (27.85% ±0.63: Fig. 4F).

Kv1.2 and Kv2.2 Localization at the AIS is Dependent on Ankyrin G and Independe Cytoskeleton

To identify the mechanisms underlying the localization of Kv1.2 and Kv2.2 to the AIS, we first evaluated the resista property common to other proteins concentrated in the AIS, such as ankyrin G and the protein recognized by 14D4 DIV hippocampal neurons, Kv1.2 (Fig. 5A) and Kv2.2 (Fig. 5D) expression was still evident in the AIS after extract identified by 14D4 and ankyrin G immunostaining, respectively (Fig. 5). This suggests that potassium channels in t scaffolding proteins that are resistant to detergent extraction, such as ankyrin G.

We also assessed whether the localization of Kv1.2 and Kv2.2 in the AIS was dependent on the integrity of the ac the structure and function of the AIS [13], [64], and to maintain the structure and neurochemical features of the cis disrupted in neurons by exposing them to cytochalasin D (5 μM) from 15 to 17 DIV, as witnessed by the altered pa control neurons (Fig. 6I and L). However, neither the expression nor the distribution of Kv1.2 or Kv2.2 was altered (see arrows in Fig. 6). Hence, the polymerized state of actin microfilaments does not actively influence the distribu AIS.

We next investigated the role of ankyrin G in the retention of Kv1.2 and Kv2.2 potassium channels, nucleofecting n ankyrin G shRNA and GFP, and maintaining them in culture until 18 DIV. The absence of ankyrin G expression in th shRNA was verified by ankyrin G immunostaining. No ankyrin G expression was detected in any of the processes of GFP-positive ankyrin G shRNA-nucleofected neurons (Fig. 7J). Moreover, another AIS marker, recognized by 1 G shRNA-nucleofected neurons (Fig. 7D), as described previously [72]. By contrast, ankyrin G expression persiste scrambled shRNA plasmids (Fig. 7G). In parallel with the loss of ankyrin G and 14D4 staining, no tethering of Kv1. AIS of neurons nucleofected with ankyrin G shRNA, or in any other process emanating from the soma (Fig. 7B, E, neurons (distinguished by GFP staining), Kv1.2 (Fig. 7A–C) and Kv2.2 (Fig. 7G–I) expression remained localized i observed in non-nucleofected neurons (Fig. 2 and 3). Data from three independent experiments showed that the p fell significantly ($p \leq 0.001$), from 16.23 (±2.65%) in scrambled shRNA-nucleofected neurons (total number of nucle ankyrin G-nucleofected neurons (106 nucleofected neurons. Similarly, the clusters of Kv2.2 immunostaining observ ankyrin G, while Kv2.2 expression in the soma was unaffected (Fig. 7 J–L). The mean percentage of neurons expr 71.83 (±1.88%) in scrambled shRNA nucleofected neurons (111 nucleofected neurons) to 1.89 (±0.97%) in shRNA nucleofected neurons). These results, strongly suggest that Kv1.2 and Kv2.2 tethering and clustering at the AIS is cytoskeleton.

Discussion

The present findings indicate that in contrast to the early expression of voltage-gated sodium channels (VGSC) an Kv2.2 subunits are first tethered at the AIS of cultured hippocampal neurons after 10 days *in vitro* (DIV). Furtherm distinctly in the AIS, with each subunit largely restricted to distinct populations of neurons. Our results show that th

AIS is resistant to detergent extraction and like other AIS proteins, it is dependent on the presence of ankyrin G. M subunits in the AIS is not affected by the disruption of the actin cytoskeleton.

AIS Maturation

Cultured hippocampal neurons are widely used as a model to study the development of neuronal polarity [55]. This followed by its subsequent elongation and the development of functionally specialized subdomains, including the AI transport and precise spatial and temporal localization of membrane and cytoskeletal components. Among the first are ankyrin G [56], [57], the protein recognized by 14D4 immunostaining [58], [71] and casein kinase 2 [72], which Ankyrin G is responsible for the accumulation of other structural and functional proteins to the AIS, including VGSC shortly after ankyrin G accumulation in this region [57], [58]. We found that the ankyrin G-dependent targeting and the AIS [48], [49] was a relatively early event in AIS maturation (3–6 DIV), although it occurred after VGSC expres and Kv2 channel expression, which begin to concentrate at the AIS during the second week *in vitro* (10 DIV). The of cultured hippocampal neurons is concomitant with the appearance of gephyrin and $GABA_A$ receptor subunits at organelle, which is involved in Ca^{2+} regulation and reaches neurochemical maturation during the second week *in vi* determine whether these late events in AIS maturation are coordinated with the expression of Kv1 and Kv2 channe axonal domain, and to identify the functional consequences of AIS maturation on action potential generation and th

Kv Channel Distribution in the AIS

While the uneven distribution of different Kv channel types is required for proper neuronal function, the specific cell channel proteins has not been fully elucidated [73], [74]. We observed a distinct distribution for Kv1, Kv2 and Kv7 or KCNQ channel is expressed in the AIS of different neuronal types, including the rodent adult hippocampus and c [49], [50], [51]. We found that during the first three weeks of *in vitro* development, the Kv7.2 subunit was homoge in the vast majority of hippocampal neurons. This is consistent with the homogeneous distribution of ankyrin G thro of Kv7.2 and Kv7.3 are required for their localization to the AIS [46], [48], [49].

In contrast to Kv7 channels, the Kv1.2 subunit of Kv1 channels was restricted to the distal region of the AIS. AIS c segregation of different VGSCs and the enrichment of the distal AIS with Kv1 channels, and it has been linked with regions in generating and back-propagating APs, respectively [13], [17], [18], [27], [28], [29], [30], [31], [32], [33]. the distal versus the proximal AIS is only observed in certain neuronal types, probably reflecting electrophysiologic These include neocortical pyramidal cells in layer 2/3, interneurons, CA1 pyramidal neurons and retinal ganglion ce pyramidal neurons in layer V of the neocortex or in the CA3 region of the hippocampus [17], [18], [28], [35]. In our expressed Kv1.2 at the distal AIS at 21 DIV. This percentage may reflect the proportion of CA1 pyramidal neurons expression in the AIS of the neuronal population corresponding to the 60% of neurons that do not express Kv1.2.

Kv2 delayed rectifier channels include those comprised of Kv2.1 and Kv2.2 subunits, although they can also form h subfamilies (Kv5, 6, 8 and 9) [76]. Kv2 channels regulate excitability in hippocampal and cortical neurons rather tha potential repolarization [37], [39], [40], [41], [42], [43], and they are mainly distributed in clusters of around 1.3 mic dendrites of neocortical and hippocampal neurons [38], [40], [77], [78], [79], [80], [81], [82], [83], [84]. Clusters of contact points [85] being also coincident with membrane zones associated with subsurface reticulum cisterns, kno Kv2.1 clusters overlap with clusters rich in ryanodine receptor Ca^{2+} release channels and the luminal Ca^{2+} binding of Kv2 channels in Ca^{2+} regulation [37], [82], [85]. We found that in addition to this somatodendritic domain, Kv2.2 cells, mainly in the proximal AIS. Hence, Kv2.2 subunits may contribute to the maintenance of the AP amplitude in potential during high frequency firing, as occurs in neurons of the median nucleus of the trapezoid body [45]. The A composed of stacks of smooth endoplasmic reticulum cisterns. The outermost of these elements is in apposition t IP_3R-expressing microdomains [70]. However, no spatial overlap appears to occur between Kv2.2-expressing AIS microdomains (unpublished observations), suggesting that AIS Kv2 channels are not involved in the IP_3R1-mediate

The clustering of Kv2.2 at the AIS described here is consistent with that of the Kv2.1 subunit in hippocampal neuro Whether Kv2.2 colocalizes with Kv2.1 in the AIS clusters remains unknown. At 21 DIV, Kv2.2 clusters were obser neurons, and a similar proportion of neocortical pyramidal neurons exhibited Kv2.2 somatodendritic immunostaining and Kv1.2 subunits at the AIS in cultured hippocampal neurons was mutually exclusive. It remains unclear whether differences in neuronal type within the mature hippocampal formation, or alternatively, a lag in *in vitro* Kv1.2 or Kv2

Mechanisms Mediating AIS Localization of Kv Channels

In recent years, several studies have described mechanisms responsible for the concentration of ion channels at d protein interactions, and have identified amino acid motifs involved in these interactions. However, the functions of and the mechanisms responsible for channel trafficking and clustering at the AIS have yet to be fully characterized

In the AIS, the presence of ankyrin G and its interaction with the actin cytoskeleton through βIV spectrin is critical such as VGSCs, the adhesion molecules neurofascin-186 and NrCAM, and Kv7 potassium channels [16], [46], [48 Accumulation of the latter occurs through direct binding of Kv7.2 and Kv7.3 subunits to ankyrin G via an ankyrin G [48], [49], [91].

Proteins that form complexes with Kv1 subunits (Kv1.1, Kv1.2 and Kv1.4) include Caspr2, TAG-1 and ADAM22, ai localization of Kv1 in the AIS is dependent on the presence of PSD93/Chapsyn-110 and on PDZ domain interactior may also be involved [74], [94]. We found that the accumulation of Kv1.2 subunits in the AIS of cultured hippocamp cytoskeleton and was dependent on the presence of ankyrin G at the AIS, as these subunits were absent in neuro evidence for a direct interaction between ankyrin G and Kv1.2 has been reported [20], our data indicate that the p development, and for the acquisition and/or maintenance of Kv1.2 and Kv2.2 expression at the AIS. This view is in knockdown resulted in the loss of ankyrin G-interacting proteins, such as Na^+ channels, βIV spectrin and neurofas casein kinase 2α, IP_3R1, annexin 6, synaptopodin and α-actinin immunostaining at the AIS [69], [70], [72].

The diverse mechanisms involved in Kv2 channel clustering, including that which occurs at the AIS, remain to be fu dynamic structures, the maintenance and localization of which may depend on the presence of a targeting motif kn (PRC) signal [81], or on their interaction with scaffold proteins [95]. Kv2.1 clustering and the voltage-dependence phosphorylation in response to both neuronal activity-induced Ca^{2+} influx and Ca^{2+} release from internal stores [37 are mobile, although their mean diffusion coefficient is lower than that outside the clusters, suggesting that the clus cytoskeleton [97], [99]. Kv2.1 clusters are reported to favor cell surface regions not associated with phalloidin-pos depressions in the cortical cytoskeleton corralled by a high density of cortical actin filaments [99], [100]. According hippocampal neurons has been reported to either increase Kv2.1 cluster size [99] or induce complete cluster disso more stable than those found in the soma [44]. Moreover, although no measurements of cluster size were perform were not affected by disrupting the actin cytoskeleton with cytochalasin D, which disrupts the diffusion barrier of th asymmetric distribution of other AIS proteins and lipids [64], [65], [101]. Together with the absence of Kv2.2 cluste neurons, these findings suggest that in addition to the actin cytoskeleton, other as yet uncharacterized molecular in channel clusters in the AIS.

Author Contributions

Conceived and designed the experiments: AM JJG. Performed the experiments: DS. Analyzed the data: DS AM J. JD JJG AM. Wrote the paper: DS AM JJG.

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